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# Estimating the Heritability of Plasticity of Thermal Tolerance and its Application in the Restoration of Endangered Caribbean Coral

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# Estimating the Heritability of Plasticity of Thermal Tolerance and its Application in the Restoration of Endangered Caribbean Coral

by

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## Abstract

Rising surface temperature poses a threat to the world's coral reefs and has been the cause of many mass bleaching events. Since coral colonies are sessile, survival of individual colonies may require them to acclimate to elevated water temperatures. Phenotypic plasticity, or the ability of an individual to express different phenotypes to survive in different environments, has been suggested as a critical first step that can buy a population time to evolve important adaptations. This chapter investigated two questions related to plasticity in high temperature tolerance in the endangered Caribbean coral, *Acropora cervicornis*: 1) Are coral colonies capable of acclimating to elevated water temperatures? 2) Do coral genotypes differ in their ability to acclimate to thermal stress (phenotypic plasticity of thermal tolerance)? We subjected *A. cervicornis* clones to different acclimation temperatures to identify the presence of plasticity in thermal tolerance, and estimated the broad ($H^2$) and narrow-sense ($h^2$) heritability of both thermal tolerance and plasticity of thermal tolerance. The study found that colonies exposed to a previous, short-term heat stress survived a long-term heat stress significantly longer (141 hours) than those that were acclimated at ambient temperatures. The amount of plasticity was found to depend on genotype, but not necessarily to additive effects of genotype, with $H^2 = 0.19$ and $h^2 = 0$. This suggests *A. cervicornis* lacks the capacity to evolve greater plasticity in thermal tolerance over a short period of time. However, our estimates may underrepresent the true $h^2$ in this population, and there are other factors such as epigenetic response and holobiont composition that may play a significant role in plasticity of thermal tolerance.

## Introduction

Anthropogenic activities have led to increased concentrations of $CO_2$ in the atmosphere, creating a greenhouse effect that is projected to increase global surface temperatures 1-4 $^oC$ within the century (Hoegh-Guldberg et al., 2007, IPCC 2014). Increases in surface temperature pose a threat to coral reefs because coral species tend to live at or near their thermal threshold (Jokiel and Coles 1990; Berkelmans & Willis, 1999; Hoegh-Guldberg, 1999). Elevated temperatures are the cause of many mass-bleaching events, where corals expel their symbiotic dinoflagellates as increased heat damages photosystem II and causes spillover of reactive oxygen species into coral tissues (Weis, 2008) sometimes leading to mortality (Glynn, 1983; Porter & Meier, 1992; Blaird & Marshal, 2002).

To combat rising surface temperatures, corals must either relocated to cooler waters, acclimate to elevated temperatures, or evolve higher thermal thresholds. Relocation requires motility, which is only possible in a coral's larval stage and is limited by a variety of oceanographic and physiological factors (Pechenik, 1990; 1987). Evolution requires multiple generations for selection to alter the population, and with one-third of corals facing extinction due to climate change, (Carpenter et al., 2008) evolution may not occur fast enough to stop mass extinction. Acclimation therefore may be the most probable short-term solution to rising surface temperature, as it may function as an initial step that buys time for adaptation to occur (Chevin & Lande, 2010; Snell-Rood et al., 2018).

Phenotypic plasticity is a form of acclimation which improves the probability of survival in populations under stressful conditions without evolution (Pigliucci, 2005). Phenotypic plasticity, or the ability of an individual to express different phenotypes to survive in different environments (Ghalambor et al., 2010) is crucial for sessile organisms since it can increase

survivability in species incapable of moving to better conditions (Sultan, 1995). Phenotypic plasticity is typically observed in organisms that occupy areas with highly variable environmental conditions and where the ability to quickly respond to environmental changes is favorable (Via & Lande, 1985).

Previous studies have shown prior exposure to elevated temperatures improves the survivability of some coral species to future heat stresses (Middlebrook et al., 2008; Oliver & Palumbi 2011; Maynard et al., 2008). It is unknown whether phenotypic plasticity in thermal tolerance is widespread among coral species, or if it is a trait held only by a few. Furthermore, while host genotype significantly influences thermal tolerance (Fitt et al., 2009; Weis, 2010; Csazar et al., 2010, Kenkel et al., 2015), it is unknown whether coral genets differ in their ability to acclimate to temperature changes. If variability in plasticity of thermal tolerance is found among genotypes, restoration managers may choose to propagate specific corals that are more plastic in their tolerance to thermal stress, effectively using assisted evolution to increase the frequency of plastic genotypes and bolstering the resilience of outplanted coral (Van Oppen et al., 2015).

Two mechanisms have been hypothesized to explain phenotypic plasticity in coral. First, coral may alter gene expression patterns that result in physiological changes in response to environmental shifts. When experiencing thermal stress, some species increase expression of genes that transcribe antioxidant (Császár et al., 2009), anti-apoptosis, and symbiont-recognition proteins (Bellantuono et al., 2012). Increased production of antioxidants may reduce damage from free-radicals activated by photosystem II. Anti-apoptosis proteins may prolong cell life while under stress, and symbiont recognition proteins may serve to reinforce the bonding of symbionts in the endoderm, reducing the likelihood of bleaching (Bellantuono et al., 2012).

Other species have been observed increasing expression of heat-shock and chromoproteins (Seneca et al., 2010). Heat shock proteins help maintain conformation among structural proteins and important enzymes, while chromoproteins assist in absorbing excess solar radiation. Actin and collagen are also upregulated, suggesting that cellular integrity becomes a priority when threatened with excess heat (Kenkel et al., 2013). The same study also discovered the downregulation of genes coding for adenosine kinase and carbonic anhydrase, two enzymes important for growth and calcification respectively, suggesting there may be a tradeoff in growth and maintaining cell integrity while under thermal stress.

Second, changes in the amount, type or combination of symbiotic dinoflagellates may play a role in responses to temperature stress. Symbiont clade influences thermal tolerance in coral (Berkelmans & Van Oppen, 2006; Howells et al., 2012; Oliver & Palumbi, 2011), and populations that reside in areas with high average temperatures typically contain thermally tolerant zooxanthellae (Baker et al., 2004). Populations that lack thermally tolerant symbionts may be able to acquire them in a process known as "symbiont shuffling" (Meiog et al., 2007) where background populations of thermally resilient symbionts are retained within the coral's tissues while less tolerant clades are expelled (Cunning et al., 2018; Silverstien et al., 2017). Some corals have been observed collecting new symbiont clades after bleaching, possibly providing an adaptive mechanism to increase thermal tolerance by acquiring tolerant symbionts from the surrounding reef (Boulotte, et al., 2016; Byler et al., 2013).

Shallow reef zones exhibit high variability in seasonal water temperature (Leichter et al., 2006) suggesting that corals residing near the surface may require a significant amount of plasticity in thermal tolerance. One coral species typically found within 10 m of the surface is the branching coral *Acropora cervicornis* (Tunnicliff, 1981; Rützler & Macintyre, 1982*)* It's easily

fragmented skeleton and proclivity for thermally variable environments make it a suitable candidate for investigating the phenotypic plasticity of thermal tolerance.

This project addressed two questions regarding the acclimation ability to thermal stress in the endangered Caribbean coral, *Acropora cervicornis*: 1) Does short-term exposure to heat stress result in greater survival during a second heat stress? 2) To what extent are differences in the ability to acclimate to thermal stress (phenotypic plasticity of thermal tolerance) due to differences in coral genotype? Answering these questions will aid conservationists by optimizing the effectiveness of coral reef recovery projects and advance the understanding of corals' capacity to acclimate to a changing climate.

## Methods

### *Coral Collection*

A total of 12, ~15 cm long fragments from each of 20 genotypes (240 fragments) of *A. cervicornis* were collected at random from surplus stock in the Coral Restoration Foundation's (CRF) Tavernier nursery. The genotypes were fragmented offshore and left in the nursery to recover for approximately one month. After the recovery period, the fragments were transported to the Keys Marine Laboratory (KML) and allowed to recover for 3 days at ambient temperature (~26 ºC) in a pair of 492 L raceways with shading set to mimic light at 5 m, approximately the same depth as the Tavernier nursery. Fragments were arranged in a randomized grid design and placed on eggcrate ceiling panels at the bottom of each tank to elevate the fragments from the raceway floor and to facilitate raceway maintenance when necessary.

*Treatment Allocation*

Coral fragments were divided into twelve 151 L raceways so that 1 fragment of each genotype was allocated to a raceway, for a total of 20 fragments per 151 L raceway. Once dispersed to the appropriate raceways, the fragments underwent a four-day acclimation period at one of two temperatures, ambient (27 ± 1 ºC ) or elevated (30 ± 1 ºC) with 6 raceways maintained at ambient conditions and 6 raceways heated to elevated conditions. The acclimation period was followed by a five-day recovery period at ambient temperature after which the fragments spent up to 30 days in a long-term exposure at either ambient or elevated temperatures. The twelve raceways were evenly divided into four different experimental treatments that each followed the same overall design. These treatments included: 1) ambient temperature acclimation followed by ambient long-term temperatures (AA), 2) ambient temperature acclimation followed by elevated long-term temperature (AE), 3) elevated temperature acclimation followed by ambient long-term temperature (EA), and 4) elevated acclimation followed by elevated long-term (EE). Ambient temperature over the course of the experiment was ~27 ºC while elevated temperature treatments were 30 ºC and 32 ºC for the acclimation and long-term periods, respectively. For the acclimation period at elevated temperature, water was heated gradually over the course of 24 hours, maintained at 30 ºC for 48 hours, and then brought back to ambient conditions over an additional 24 hours. The long-term elevated temperature treatments were treated similarly with temperature increased over a 24-hour period to 32 ºC and maintained at that temperature for the duration of the experiment.

*Monitoring*

To monitor coral survival and health, the maximal photosynthetic efficiency (PSII quantum yield) was measured in each fragment using pulse amplitude modulated fluorometry (PAM) every 12 hours (~7:30 am and ~7:30 pm). Previous work by Rasher & Hay (2010) suggests that Fv/Fm scores of <0.2 correspond to severe bleaching and mortality. Any fragment that scored <0.2 Fv/Fm for two consecutive time periods was investigated under a microscope to verify that coral tissue had sloughed off the skeleton. Time of death was considered the time period at which all tissue had sloughed off the skeleton. Water temperature was measured using a YSI Pro Plus Water Quality Instrument (Fondreist Environmental, Fairborn Ohio, USA), with 7 temperature readings taken each day between the hours of 7:30 am and 5:30 pm. Water in experimental tanks was heated through two mechanisms. First, KML's seawater system was used to elevate temperatures up to 30 ºC, but a Hygger 500w Titanium Aquarium Heater (Shenzhen City, Guandong Province, China) was required to increase water temperature above 30 ºC for the long term exposure in AE and EE treatments. Heaters were suspended near the surface in the center of raceway in order to reach temperatures above 30 ºC. A 1-way ANOVA (IBM SPSS Statistics 25) was used to determine significant differences in lifespan among treatment groups. ANOVA was also used to determine differences in Fv/Fm among treatments before and after initial heat acclimation.

*Measuring Plasticity*

To measure plasticity, or acclimation ability, 3 fragments of each genotype were allocated into the 4 groups listed above (AA, AE, EA, EE). We quantified the overall plasticity of each genotype as a change in lifespan (LS), using the following equation:

Amount of Plasticity = (LS AA – LS AE) – (LS EA – LS EE)

Where LS represents the mean lifespans of the fragments in the AA, AE, EA, and EE treatments, respectively. The difference between LS AA and LS AE provides an estimation of the effect of elevated temperature on lifespan of coral fragments that were acclimated to ambient temperature. The difference between LS EA and LS EE estimates the degree to which elevated temperature decreases lifespan of fragments that were acclimated at an elevated temperature. The difference between the two groups (LS AA-LS AE and LS EA-LS EE) gives the degree to which lifespan at elevated temperature is affected by differences in acclimation temperature.

Using the equation above, all pairwise comparisons of lifespan differentials of the fragments acclimated at ambient temperature and those acclimated at elevated temperature were made to estimate the average plasticity of a genotype (n = 3 x 3 = 9 pairwise comparisons per genotype). Positive values of plasticity represent better performance when acclimated at elevated temperatures which would suggest acclimation, while negative values represent better performance when acclimated at ambient temperatures which would suggest the acclimation period induced stress that was not overcome during the rest period.

For the purposes of this study, the thermal tolerance of a genotype will be defined as the difference in LS of fragments exposed to elevated temperatures and those that remained at ambient temperatures. This leads to a pair of thermal tolerance estimates, including thermal tolerance when acclimated at ambient temperatures (AA - AE; hereafter ambient tolerance) and thermal tolerance when acclimated at elevated temperatures (EA - EE; hereafter elevated tolerance). For each tolerance estimate the lifespan differential was estimated for each fragment

in the treatment group and these estimates were then averaged for each genotype. By this definition, genotypes with high thermal tolerance will be those with the smallest average difference between the lifespan of treatment fragments and control fragments.

*Heritability Estimates*

The heritability of plasticity, which estimates the degree to which differences in acclimation ability is determined by differences in genotype, can be subdivided into Broad ($H^2$) and Narrow Sense ($h^2$) heritability. $H^2$ was estimated using the clonal methods described by Csaszar et al. (2010). Heritability was estimated under the random-effect ANOVA model, more specifically, a general linear mixed-effects models (GLMM) fitted by restricted maximum likelihood (REML). Variance in lifespan among genotypes provides an estimate of genetic variance ($V_G$), while variance in lifespan among fragments of the same genotype provides an estimate of environmental variance ($V_E$). The sum of $V_G + V_E$ yields an estimate of phenotypic variance (Vp) and $H^2$ can then be estimated according to:

$$H^2 = V_G / Vp.$$

Standard error was evaluated by the parametric bootstrapping method, where bootstrap samples were simulated from a Gaussian distribution whose parameters are estimated based on the real sample (Nakagawa & Schielzeth, 2010; Stoffel et al. 2017).

Narrow sense heritability was estimated by regressing the phenotypic difference in lifespan among fragments with pairwise relatedness between fragments in a linear model as outlined by Ritland (1996). These regressions provide an $R^2$ value that approximates the narrow sense heritability of phenotypic plasticity of thermal tolerance. 15 microsatellite markers were used to estimate relatedness among genotypes in order to estimate narrow-sense heritability. One

fragment from each genotype was initially preserved in 70% ethanol for genetic analysis. Subsamples of tissue and skeleton (~1 g) were immersed in “Chaos” buffer extraction (4.5 M guanidine thiocyanate; 2% N-lauroyl sarcosine; 50 mM ethylenediaminetetraacetic acid [EDTA], pH 8; 0.1 M 2-mercaptoethanol) and incubated at room temperature for 5 days. Total DNA was extracted using a magnetic bead protocol per manufacturers instructions (AMPure XP, Agencourt). Corals were then genotyped at the 15 microsatellite loci described by Baums et al. (2005) and Baums et al. (2009) utilizing the published methodology. After amplification, samples were sent to the University of Florida where fragments were processed using a Thermo Fisher 3730XL DNA Analyzer.Alleles of the 15 microsatellite loci were visually inspected using GeneMarkerV2.7.0 and genotypic data was uploaded to MARK (Ritland, 2004) to calculate pairwise relatedness of the genotypes using the Ritland relatedness estimator.Various estimators of relatedness exist, each of which has different assumptions and performs best under different circumstances (Van de Casteele et al. 2001; Russello & Amato 2004; Csillery et al. 2006). Since it is unknown which estimator best fits *A. cervicornis* additional estimates of relatedness were calculated using COANCESTRY (Wang, 2011) including: Trioml, Wang, Lynchli, Lynchrd, Quellergt, and Dyadml. This allowed us to determine how consistent the estimates of $h^2$ are when utilizing different estimators of r. Average performance is suggested to be strongest in lynchrd and ritland (Russello & Amato 2004; Coltman 2005), however, Lynchrd, Ritland and Quellergt exhibit higher bias than other estimates with small sample size and tend to produce illogically large values for r when allele frequencies approach 0.5 or 0.25 in a locus (Wang, 2002). Wang (2002) supports Lychrd as the best estimator if sample size is large and loci are not highly polymorphic, but Wang and Trioml may be more accurate when considering highly polymorphic loci (Wang 2011). Furthermore, Ritland (1996b) suggests using Ritland for

populations where the variance in relatedness is expected to be high, which is not guaranteed in individuals harvested from a nursery setting. Standard error of $h^2$ was estimated as the standard error of each linear regression between relatedness and phenotypic difference calculated in SigmaPlot (Systat Software, San Jose, CA).

**Results**

Heat acclimation successfully decreased average Fv/Fm yields among EA and EE groups (Figure 1)($F = 116.24$, $df=179$, $p < 0.01$) ($F = 100.07$, $df=179$, $p < 0.01$). Average yield initially varied according to genotype, with fragments of genotype U38 exhibiting higher average yields than U6 ($F = 1.76$, $df=19$, $p = 0.039$). After heat acclimation, average yield returned to ambient levels $T(119) = -0.87$, $p = 0.39$) and differences in average yield among genotypes changed, with average yield among genotype U38 significantly lower compared to average yields among U104, U11, and U40 ($F = 2.75$, $df = 19$, $p = 0.001$).

Mean lifespan differed significantly among treatments ($F= 212.78$, $df=3$, $P<0.001$) with lower lifespans in the AE and EE treatments while all fragments in the AA and EA treatments survived for the duration of the experiment (Fig. 2). While acclimation temperature did not affect survival of fragments maintained at ambient temperature for the duration of the experiment, it had a significant effect on survival at elevated temperature. Fragments that were acclimated at the elevated temperature (EE) survived an average of 141 hours longer than those acclimated at ambient temperatures (AE)suggesting that previous exposure to heat can increase lifespan at elevated temperature ($F=32.7$, $df=3$, $P=0.006$).

The amount of plasticity in thermal tolerance was not consistent among genotypes ($F=45.05$, $df=19$, $P<0.001$, Fig. 3A) and yielded a broad sense heritability estimate of $H^2= 0.19$

(SE=0.0754). While the majority of genotypes showed increased survival at high temperature when given a prior heat stress, genotype U107 showed lower average lifespan among fragments acclimated to elevated temperature than those acclimated at ambient temperature. Even among those genotypes where a high temperature acclimation led to increased survival at high temperature, this increase ranged from ~40 hours in genotype U37 to >250 hours in genotype M21. A Tukey's post-hoc revealed genotypes U11, U106, and M21 as having significantly greater mean plasticity than genotype U107 ($p<0.05$). Estimates of narrow sense heritability ($h^2$) of plasticity in thermal tolerance were essentially the same for all seven models of relatedness. All estimates ranged from 0.003-0.013, and none were significantly different from zero (Table 1).

To determine if heritability of plasticity is correlated with thermal tolerance at either ambient or elevated temperature acclimation, we tested for differences in lifespan among genotypes under the two different acclimation temperatures separately. Fragments acclimated to ambient temperatures exhibited no significant difference in survival time among genotype ($F=17.959$, $df=19$, $P=0.525$, Fig. 3B) resulting in a broad sense heritability of ambient tolerance of $H^2=0.00$ (SE=0.0785). Similarly, no significant difference in thermal tolerance was found among fragments acclimated at elevated temperature ($F=19.069$, $df=19$, $P=0.452$, Fig. 3C) again yielding a broad sense heritability of elevated tolerance of $H^2=0.00$ (SE=0.0785). Narrow sense heritability estimates for both ambient and elevated tolerance of fragments also approximated zero (Table 1).

Plasticity estimates were then regressed against both ambient tolerance and elevated tolerance in separate analyses to determine if the amount of plasticity shown by a genotype could be predicted by either of the tolerance estimates. Ambient tolerance explained little of the

variance observed in plasticity among genotypes ($R^2 = 0.036$, $F(1,18) = 0.676$, $p = 0.42$, Fig. 3A), while elevated tolerance explained a significant proportion of the variance observed in plasticity among genotypes ($R^2 = 0.727$, $F(1,18) = 48.10$, $p < 0.400$ Fig. 3B).

## Discussion

### *Thermal Tolerance*

Heritability for thermal tolerance among corals acclimated to ambient and elevated temperature were estimated as 0. Neither groups acclimated to ambient or elevated temperatures showed significant differences in thermal tolerance among genotype after exposure to long-term heat stress. However, a regression of elevated tolerance and plasticity shows that plasticity is mostly driven by differences in tolerance at elevated acclimation, with corals exhibiting the highest thermal tolerance after acclimation to elevated temperatures displaying the highest plasticity of thermal tolerance.

Our heritability of thermal tolerance estimates were significantly lower than estimates conducted from other studies. Yetsko (2018) calculated the heritability of *A. cervicornis* acclimated to elevated temperature as $H^2 = 0.52$. Both Dziedzic (2019) and Dixon et al., (2015) estimated the heritability of thermal tolerance of corals acclimated to ambient temperatures, with Dixon et al., (2015) estimating broad sense heritability of thermal tolerance of *Acropora millepora* as $H^2 = 0.87$ and Dziedzic estimating the narrow sense heritability of thermal tolerance of *O. faveolata* as $h^2 = 0.58$. These values greatly exceed our estimates of narrow and broad sense heritability of thermal tolerance for *A. cervicornis* at elevated and ambient conditions. It is probable that *A. cervicornis* differs in its heritability from other species, since heritability is dependent upon allele frequencies which inherently vary from species to species (Falconer &

MacKay 1996). However, the large degree to which our estimates deviate from similar studies suggests our study underestimates the true heritability of thermal tolerance in *A. cervicornis*.

The low estimates of broad-sense heritability in this experiment were due to high variability in lifespan within individual genotypes, which in turn were attributed to environmental factors due the fact that clones are genetically identical. One environmental factor that may explain variability in lifespan within genotypes is prior exposure to different light and temperature regiments in the CRF nursery. Corals were fragmented and arranged on nursery trees randomly, and some fragments of the same genotype may have been hung on top of the tree while others were placed on the bottom. Tree tops are suspended approximately 5 m below the surface, while the lowest branches are suspended approximately 8 m deep. Corals hung on the tops of trees may experience higher irradiance than individuals hung on the lower branches, which may induce differential gene expression among fragments. Changes to small scale environmental gradients have been shown to alter the transcriptome of coral clones and even different polyps within the same colony (Bay et al., 2009). Further research has shown gene methylation differs based on the location of the coral tissue on the colony and may account for variability in bleaching colonies during thermal stress (Durante et al., 2019). Therefore, the ability for polyps to rapidly acclimate to elevated temperatures may depend on the coral's life history as well as the origin of the fragment from the colony.

*Plasticity of Thermal Tolerance*

Fragments of *A. cervicornis* that were exposed to a short-term heat stress displayed a considerably longer average lifespan (141 hours) than fragments that were acclimated to ambient conditions. While no other studies have used lifespan as a metric for acclimation, this result is supported by other studies that used bleaching as a metric to investigate acclimation to thermal

stress among corals (Maynard et al., 2008; Middlebrook et al., 2008; Bellantuono et al., 2012). The ability to acclimate to elevated temperatures varied significantly among genotypes, yielding a broad sense heritability estimate of the plasticity of thermal tolerance of 0.19. This means 19% of the variation in plasticity of thermal tolerance can be attributed to differences among coral genotypes. Narrow sense heritability of plasticity in thermal tolerance averaged < 0.008 among all estimates, suggesting most of the variance in genotype may not be additive, but rather due to dominance or epistatic variance. Low narrow sense heritability suggests this population of *A. cervicornis* has a limited capacity to counter projected ocean surface temperatures via natural selection. This is because additive genetic variance is the only proportion of genetic variance that can be acted upon by selection. If selection cannot act upon the genetic variance among *A. cerviconris*, plasticity will not evolve in the species.

It is possible, however, our $h^2$ estimate is lower than the true heritability value due to the relatedness estimates used in this study. Narrow sense heritability estimates rely heavily upon the number of microsatellite markers used and the degree of variation in relatedness among genotypes (Ritland 1996). Our mean relatedness estimate using the Ritland estimator was negative (-0.097), which according to Ritland, may be due to using an insufficient number of loci, or if the population consists predominantly of individuals of similar relatedness, or completely unrelated. The 15 loci used in this study meet the parameters specified by Ritland (1996). However, the pairwise relatedness estimates are all zero except for clones. Therefore, it is probable that our narrow sense heritability estimate for plasticity of thermal tolerance was likely skewed to low relatedness among genotypes.

Hill et al., (2008) offers further evidence that our study underestimates $h^2$ of plasticity of thermal tolerance. Hill et al. determined that $h^2$ is often approximately half of $H^2$ estimates in

studies for morphological traits in laboratory animals and human twins. Even if $h^2$ was estimated at approximately half of our estimate of $H^2$, the narrow sense heritability of plasticity of thermal tolerance would still be relatively low, at $h^2 = 0.09$ for plasticity and essentially $h^2 = 0.0$ for thermal tolerance. However, low additive variance doesn't necessarily ruin a population's ability to evolve when faced with new environmental stressors. Cheverud & Routman (1996) discussed how epistasis can become additive genetic variance within a population that has undergone a population bottleneck. Willis and Orr (1992) determined that population dominance variance can become additive under population bottlenecks as well. This suggests that rescue of additive variance can occur if enough genetic variation exists within the population.

To our knowledge, no studies have investigated the heritability of phenotypic plasticity among corals. With no other studies to compare to, it is possible that our study provides an accurate estimate of $H^2$ of plasticity of thermal tolerance. It is intuitive that different genotypes may exhibit different patterns in gene expression, and several studies have hinted this is true in at least some coral species. Kenkel & Matz (2013) investigated the plasticity of gene expression of *Porites asteroids* residing in thermally consistent and variable environments. Their study determined that corals from variable thermal environments developed an ability to dynamically regulate expression of heat stress response genes, which helped reduce bleaching following thermal stress. These effects were proposed to be due to coral genotype and not to symbiont shuffling because *P. asteroides* is a brooding coral that maintains the same symbiont clade throughout its germ line. Furthermore, gene expression patterns for cell adhesion, heat shock, symbiont recognition, and oxidative stress have been observed to vary according to genotype regardless of environment (Desalvo et al., 2008; Barshish et al., 2010; Bellantuono 2013).

*Environmental Factors that Influence Plasticity*

Other factors may account for variation within genotypes in our study. First, if individual reefs experience different temperature parameters, such as elevated seasonal temperatures, corals may acclimate to express different phenotypes resilient to their original reef. It would be expected that plastic phenotypes would arise from reefs that experience temporally consistent changes in temperature, assuming plasticity is heritable (De Jong, 1995). It is important to note the coral stock in this study were an amalgamation of individuals originally taken from reefs among the upper and middle keys, so corals from different locations may be acclimated to their original reef. Durante et al., (2019) suggests acclimatization to local stressors may be partially attributed to differential methylation, where corals are methylated differently depending on immediate environmental factors. Methylation refers to epigenetic changes, or modifications in DNA and chromatin that doesn't change the DNA sequence (Duncan et al. 2014). Addition of methyl groups to histones can decrease the tension with which the proteins wind DNA, allowing for easier access to transcription factors which increases transcription of genes that may ameliorate thermal stress. However, this same mechanism may also constrict histones, downregulating gene expression and limiting the response of the coral to thermal stress. Durante et al., (2019) found that differences in methylation can be found even within a colony, particularly between exposed branches and stalks. This may explain the high variance among fragments of the same genotype, as well as differences in genotypes originally sampled from different reefs.

Second, symbiont composition may also influence the plasticity of thermal tolerance in *A. cervicornis.* Resilient clades will extend the duration of heat stress before the coral bleaches (Sympao et al., 2008). Corals often retain multiple background clades that comprise <1% of the

relative symbiont abundance (Sogin et al., 2006; Reid and Buckley, 2009). Some corals have exhibited the ability to alter the dominant symbiont clade to best suit the current environment, a phenomenon known as symbiont shuffling (Baker et al., 2004). For example, Clade D often remains in coral tissue after a bleaching event due to the clade's resiliency to extreme temperatures and tendency to remain within its host coral despite varying temperatures (Silverstein et al., 2017). However, it is unlikely symbiont shuffling occurred in our experiment. Goulet (2006) states that most corals contain one clade of zooxanthellae, and species that retain one clade have never been observed exchanging symbionts from the environment or from cryptic subpopulations within the colony. No bleaching was observed during the initial heat stress, suggesting that the clade imported from the nursery remained within coral tissues for the duration of the experiment. If symbiont composition influenced plasticity and thermal tolerance, it would mean fragments contained different clades when they were harvested from the offshore nursery. *Acropora cervicornis* in the Florida Keys is typically dominated by clade A, however inland colonies have been found to harbor clade D (Baums et al., 2010). An analysis of symbiont composition will be necessary to determine if clade dominance changed over the course of the experiment.

In conclusion, *A. cervicornis* exhibits phenotypic plasticity of thermal tolerance. However, the heritability of plasticity of thermal tolerance is low compared to other estimates of heritability of thermal tolerance among coral species. Our estimates likely underestimate the true heritability of thermal tolerance and its plasticity due to the low relatedness estimates among the corals used in this study. Our low estimate for narrow sense heritability of plasticity suggests plasticity of thermal tolerance may not evolve among *A. cervicornis* populations at a rate fast enough to combat rising surface temperatures. However, this study likely underestimates narrow-

sense heritability due to low relatedness among genotypes. It is more likely this population exhibits some narrow sense heritability, especially since epistatic and dominance variance can transform into additive genetic variance in bottlenecked populations. Even if our estimates accurately represent heritability of plasticity, other mechanisms of acclimation are still available to *A. cervicornis.* Epigenetic traits have recently been revealed to be passed down throughout generations and may be a promising mechanism of localized acclimation. Lastly, symbiont composition plays a significant role in coral thermal tolerance and may be significant source adaptive potential among *A. cervicornis* populations.

# Chapter 2: The Application of Thermal Frontloading in the Restoration of Endangered Caribbean Coral


## Abstract

To compensate for coral losses, managers are actively rearing and "outplanting" coral fragments to replenish the colonies lost on local reefs. While these efforts have experienced some success, many outplants are lost within the first year of transplantation. This has prompted managers to investigate innovative techniques to rapidly acclimate fragments to elevated thermal thresholds through a process known as "frontloading". Frontloading is accomplished by introducing corals to an initial heat stress before outplanting. We investigated whether prior heat stress before outplanting increases the growth and viability in transplanted fragments of *Acropora cervicornis*, *Acropora palmata*, and *Orbicella faveolata*. Corals were acquired from an offshore nursery and treated to a heat stress, where temperatures were gradually increased from 26 ºC to 30 ºC for 24 hours, maintained at 30 ºC for 48 hours, then returned to 26 ºC over 24 hours. After a recovery period all fragments were outplanted to 3 different reefs at shallow (~3 m) and deep (~6 m) depths. Corals were monitored 1, 3 and 8 months after outplanting to measure changes in growth and mortality in addition to a suite of other factors including bleaching, disease, predation, and sedimentation. Our study found that corals treated with a heat stress did not grow or survive more than fragments that received no such treatment. While treatment had no effect on growth or mortality of any species, outplant depth at 6 m negatively influenced growth and mortality of *A. cervicornis* and *A. palmata*. Our study suggests that frontloading corals over a 3-day acclimation period isn't an effective technique in maximizing coral restoration, and further research will be necessary to determine if frontloading can be used to improve active restoration of coral reefs.

## Introduction

Anthropogenic climate change is expected to increase ocean surface temperature by 1-4 $^{o}$C within the century (Hoegh-Guldberg et al., 2007; IPCC, 2014). This increase in temperature poses a global threat to coral reefs because many corals live within 1-2 $^{o}$C of their thermal limit (Coles et al., 1977). Exposure to temperatures beyond their thermal limits disrupts the relationship between corals and their symbiotic zooxanthellae, causing the coral to expel their symbionts in a phenomenon known as coral bleaching (Warner et al., 1999), which often leads to coral mortality due to the loss of nutrients acquired through symbiosis (Jones, 2008). Recently, researchers have begun investigating options for coral conservation and restoration. Active restoration, or the transplant of coral fragments to reefs by managers, has been utilized as a successful tool to reclaim degraded reefs (Van Oppen et al., 2015; Naughton & Jokiel, 2001). While many restoration efforts have experienced some degree of success, outplant mortality can be as high as 50% with the highest mortality rate occurring during the first year of outplanting (Bowden-Kerby, 2008; personal communication, A. Moura, Coral Restoration Foundation). High coral mortality has contributed to a focus on assisted evolution, or the propagation of alleles among coral populations that promote thermal tolerance, or acclimation through the introduction of stress, as a means of preserving threatened coral populations (Van Oppen et al., 2015; 2017). There are several techniques available to preserve corals experiencing mortality due to climate change. First, some colonies may contain alleles that increase thermal tolerance. If tolerant genotypes are identified, managers may take fragments of these colonies and propagate them either asexually through further fragmentation, or through selective mating with other tolerant genotypes. If variation in thermal tolerance is not available, some species are capable of hybridization, with the hybrid offspring displaying elevated thermal tolerance (Fogarty, 2012).

Second, managers may inoculate coral larvae with thermally tolerant symbionts. Many coral larvae collect their symbionts from the local environment, providing an opportunity for managers to rear their own larvae with symbionts catered to their target reef. Multiple studies have confirmed that certain symbiont clades confer higher thermal tolerance to their coral hosts (Mieog et al., 2007). Furthermore, some coral species have exhibited the ability to "shuffle" symbionts from the environment or from cryptic subpopulations within their tissues to better suit current environmental conditions (Silverstein et al., 2017; Baker et al., 2004; Baird et al., 2007). Therefore, managers may spawn coral larvae and rear them in habitats with thermally tolerant symbionts, or if the species is capable of symbiont shuffling, bleach fragments and immerse them in habitats with tolerant symbionts before transferring them to the target reef.

It has been suggested that introducing corals to a prior heat stress may acclimate them to future elevated temperatures (Brener-Raffalli et al., 2018) particularly during peak bleaching events in the summer (Liu et al., 2008). This process, known as "frontloading," may reduce coral mortality by altering gene expression patterns to be more appropriate for the temperature they are expected to experience (Middlebrook et al, 2008). Acclimation is a mechanism by which phenotypic plasticity occurs and allows survival of populations under stressful conditions without evolution (Pigliucci, 2005). Phenotypic plasticity, or the ability of an individual to express different phenotypes to survive in different environments (Ghalambor et al, 2010) is crucial for sessile organisms since it can increase survivability in species incapable of moving to better conditions (Sultan, 1995). Phenotypic plasticity is typically observed in organisms that occupy areas with highly variable environments, where the ability to quickly respond to environmental changes is favorable (Via & Lande, 1985).

Outplanting success can often differ among locations due to a variety of environmental factors. One factor that may play a role in this is depth, as shallow water retains higher average temperature and irradiance than deeper reefs (Chalker, Dunlap & Oliver 1983; Oliver, Chalker & Dunlap 1983; Barnes & Chalker 1990). In general, shallow corals should have faster growth rates due to elevated light exposure (Jokiel and Coles 1977; Coles and Jokiel 1978). While faster growth is possible, there is also a synergistic effect between temperature and irradiance upon coral tissue making shallow water a more stressful environment than deeper water (Coles and Jokiel 1978). Corals experiencing chronic stress allocate fewer resources to development and often exhibit a slower growth rate (Edmunds & Davies, 1989). It may be in these circumstances, however, that frontloading has the greatest effect in reducing mortality.

In this study we investigated if frontloading increases outplanting success in endangered Caribbean species *Acropora cervicornis*, *A. palmata*, and *Orbicella faveolata*. *Acropora cervicornis* and *A. palmata* are branching shallow water corals that utilize wave action to fragment branches into clonal propagules. They are the only two branching species found in the Caribbean and are considered endangered by the IUCN (Aronson et al., 2008) having lost 90% of their biomass over the last 40 years (ABRT, 2005). Their branched morphology provides unique reef habitat which has led restoration managers to focus on their artificial restoration in the Caribbean. *Orbicella faveolata* is an endangered Caribbean mound coral (Aronson et al., 2008) that has recently lost significant portions of its population to a recent disease outbreak in the Florida Keys. Mound corals are important reef builders that contribute the most aragonite substrate to the reef. Each species is commonly found in shallow reef zones and are severely threatened by future bleaching events, making them excellent subjects for a transplant experiment. We tested two questions: 1) does frontloading influence the growth and viability of

*A. cervicornis*, *A. palmata*, and *O. faveolata* fragments outplanted on reefs in the Florida Keys? and 2) is frontloading more beneficial for corals outplanted in shallower reefs where thermal stress is more likely? This study may produce potential techniques that could improve outplanting success and will provide valuable insight into the adaptive potential of corals, and the role of human intervention during an age of climate change.

## Methods

*Collection and Treatment*

In May 2018, 10 genotypes of *A. cervicornis* were chosen from surplus stock at the Tavernier nursery managed by the Coral Restoration Foundation (CRF) off the Southern Coast of Key Largo, FL. The same was done for *A. palmata* and *O. faveolata* in May of 2019. Twenty-four fragments of each genotype were fragmented from a parent colony and isolated on separate trees within the nursery via SCUBA diving. All CRF trees were anchored in ~10 m of water with treetops reaching ~5m deep in the water column. In June of each year (2018 and 2019), the fragments were retrieved via SCUBA, and then inspected for signs of disease and to ensure similar size (~15 cm in length for *A. cervcornis,* ~15 cm in diameter for *A. palmata*, and ~2 cm in diameter for *O. faveolata*). Following inspection, the fragments were transported to the Keys Marine Laboratory (KML) in Long Key, FL. Fragments of *A. cervicornis* were separated into two 492 L raceways, so that each raceway contained 120 fragments (12 fragments per genotype). *Acropora palmata* and *Orbicella faveolata* were divided so that 120 fragments of each species were allocated to each of the aforementioned raceways, so that 12 fragments of each genotype of each species were maintained in one raceway. All fragments were allowed to recover at ambient temperature (26 ± 1 ºC) for 3 days prior to experimentation. After recovery, 120 fragments in one raceway were allocated as an ambient treatment group and were maintained at 26 (± 1 ºC),

while 120 fragments in the second raceway were allocated as a heat treatment group and subjected to heat stress over four days. The heat stress involved a temperature increase from 26 ºC to 30 ºC over the first 24 hours, after which the temperature was maintained at 30 ºC for 48 hours before returning to ambient temperature over the final 24 hours. After 3 days of recovery all coral fragments were ouptlanted over two consecutive days.

*Outplanting*

After recovery, each species was outplanted on two reefs over the course of two days. In late June of 2018 *A. cervicornis* fragments were outplanted at Carsyfort Reef (CF, 25.3139° N, 80.2794° W) and North Dry Rocks Reef (NDR, 25.11083°N 80.30444°W). *Acropora palmata* and *O. faveolata* were outplanted at a pair of reefs over two days in late June of 2019. Like *A. cervicornis*, *Orbicella faveolata* was outplanted at CF and NDR. *Acropora palmata* fragments, on the other hand, were outplanted at NDR and at Grecian Rocks Reef (GR, 25.1119° N, 80.3052° W). Each species was planted separately in individual quadrats (Figure 5) with three quadrats planted on ~3 m deep ledges, and three quadrats planted at a depth of ~6 m. Each quadrat contained two fragments of each genotype (20 total). Fragments were identified with a plastic tag denoting one fragment acclimated at ambient temperature, and one fragment acclimated at elevated temperature. Depth was included as a potential factor that may influence the success of outplants since individuals planted at shallower depths are more likely to experience the elevated temperatures and irradiance that can cause bleaching.

*Outplant Monitoring*

Outplanted coral fragments were monitored over the span of eight months for changes in growth and mortality between timepoints, with visitations to outplants occurring at 1, 3, and 8

months after outplanting. Signs of disease, predation, bleaching and sedimentation were recorded for each fragment at the same timepoint at each site. Mortality was determined by the complete loss of tissue from the coral skeleton, while growth was defined as the change in area of coral tissue between timepoints. To estimate growth, the area of each fragment during initial outplanting and at each timepoint was estimated using Coral Point Count (CPCe 4.1, Kohler, 2004) and the area of the previous timepoint was subtracted from the current timepoint. Growth at 1 month was measured by subtracting the initial area of each fragment at the time of first outplanting in late June by the area of the same fragments measured the first week in August. A Gopro Hero3 at stock setting (3648 by 2768 pixels, aspect ratio 4:3) was used to take top-down photographs of outplanted fragments, utilizing a 50 cm PVC pipe between the camera and the substrate to ensure consistent image size.

*Statistical Analysis*

To analyze mortality data, G-tests of independence were used to measure differences in the proportion of dead fragments among the four treatment groups; control and heat-acclimated fragments planted on either the shallow or deep reef ledge. An analysis of variance (ANOVA, IBM SPSS Statistics 25) was used to determine if average growth was different at each timepoint among 4 groups: Treated/Shallow, Treated/Deep, Untreated/Shallow, Untreated/Deep. Corals in the process of bleaching were compared to a bleaching scale as outlined by Siebeck et al (2006). The difference in proportion of corals exhibiting the same bleaching score, disease, predation, or sedimentation among treatment groups were calculated using G-tests of independence. All tests were performed separately for each time point.

## Results

There were no significant differences in growth associated with heat treatment in any species at any site (Figure 6) (*df*=3 F=1.78, p =0.15) (*df*=3 F=1.65, p =0.18)(*df*=3 F=1.05, p =0.37)(*df*=3 F=1.11, p =0.35)( (*df*=3 F=0.78, p =0.51) (*df*=3 F=2.74, p =0.07). However, depth did affect growth in *A. cervicornis* and *A. palmata*, but not *O. faveolata*. For *A. cervicornis*, no differences in growth were observed between depths at CF at any timepoint, but at NDR, shallow fragments showed greater growth than deep fragments at month 3 (Figure 6) (**F,** *df*=51 F=6.38 p <0.01) (Tukey's Post-Hoc p<0.05). *Acropora palmata* showed a similar pattern with greater growth of shallow fragments at one location on one date (GR at 1 month) (*df*=3 F=8.48, p <0.01) (Tukey's post-hoc p<0.05). While both *Acropora* species exhibited an increase in growth over each timepoint, *O. faveolata* exhibited lower growth between months 3 and 8, than they did during each of the two previous timespans; a pattern that was consistent at both sites.

There was no difference in mortality due to heat treatment in any of the study species, (G=0.15, p=1.00; G=0.11, p=1.00; G= 0.08, p=1.00) but depth again showed inconsistent effects. For example, *A. cervicornis* showed no difference in mortality between depths at either site at 1 month, but fragments outplanted in deep habitats exhibited higher mortality than fragments outplanted in shallow habitats in both subsequent assessments at both sites (Figure 7) (CF G=8.92, p<0.03; G=13.86, p<0.00: NDR G=13.89, p<0.01; G=18.14, p<0.01). *Acropora palmata* fragments at GR experienced elevated mortality in shallow water by 3 and 8 months (G=16.85, p<0.01; G=20.71, p<0.01) but not in the initial time point (1 month). No significant differences in mortality were observed for fragments of *A. palmata* outplanted at different depths at NDR. Mortality remained low among *O. faveolata* until month 8 at both sites and did not follow a particular trend at any timepoint. It was also noted that after one full year, all *A.*

*cervicornis* fragments outplanted on the deep ledge at both NDR and CF had died, while ~50% were still alive on the shallow ledges.

Predation was observed infrequently among all species and likely did not contribute to mortality. Two fire worms were found among two *A. cervicornis* fragments at CF on month 3 2018, with minimal (<1 $cm^3$) tissue loss from the corals. No predation was observed among *A. cervicornis* at any timepoint at NDR. One *A. palmata* fragment was found with a fireworm at NDR on month 3, 2018, with minimal (<1 $cm^3$) tissue stripped off of the fragment. No predation of *A. palmata* was observed at GR. Only one *O. faveolata* fragment was recorded deceased to parrotfish predation at CF on month 3, 2018, and no predation was observed at NDR.

White Band Disease Type-II (WBD) was observed in 12 fragments of *A. cervicornis* in CF on month 1 and in 2 fragments on month 8, but not on month 3. Presence of WBD was not related to either heat treatment or depth ($G<0.01$, $p=1.00$; $G<0.01$, $p=1.00$). No signs of WBD were observed in our fragments outplanted at NDR, although outplants not associated with the present study were observed with this disease. Disease was not observed among fragments of *A. palmata* or *O. faveolata* at either reef. As for the other factors, no signs of bleaching or sedimentation were observed among any species. However, *O. faveolata* at both sites appeared to be crowded by macroalgae on months 3 and 8, which may have contributed to reduced growth and elevated mortality observed on month 8.

**Discussion**

*Growth*

Our study investigated whether thermal frontloading increases the growth and viablity of outplanted coral, and whether these effects are more pronounced within shallow reef zones where they likely experience more stressful temperatures and irradience levels. Frontloading had

no effect upon the growth of any of the species investigated in the current study in either a positive or negative way. So while exposure to a thermal stress prior to outplanting did not allow treated fragments to outgrow control fragments, they also did not grow less.

Growth was likely influenced by several factors. Our results show *A. cervicornis* and *A. palmata* fragments outplanted at ~6 m grew less than fragments outplanted at ~3 m between the first two visitation periods. The observed pattern was expected since corals in shallow habitats experience elevated irradiance, which stimulates photosynthesis from algal symbionts (Chalker, Dunlap & Oliver 1983; Oliver, Chalker & Dunlap 1983; Barnes & Chalker 1990). The fact this pattern was observed in late Summer and early Fall is likely due to seasonal differences in temperature and irradiance, as corals have been observed to grow faster during summer months when irradiance is higher and the days are longer (Jokiel and Coles 1977; Coles and Jokiel 1978). Less growth observed at the last timepoint relative to the first two visitations may also be contributed to fragment size, as smaller corals calcify faster, and as corals grow they naturally reduce calcification rates (Buddemeier & Kinzie 1976).

*Orbicell faveolata* was unique in that the third visitation yeilded the smallest increase in size. Given that the time between the second and third visitation is the longest in this study (110 days) the growth between these two timepoints was expected to be the greatest. While slower growth may be due to lower temperature and irradiance during the winter months as stated above, part of the reduced growth rate may be attributed to competition with macroalgae. Visitations in October and March revealed many fragments surrounded by clumps of algae belonging to the genus *Padina,* particularly among quadrats in NDR. Crowding of this fleshy brown algae may have reduced the corals capacity to acquire sunlight, thus reducing the amount of food allocated for growth. As far as we know there is no data published showing *Padina*

exhibiting allelopathic tendancies towards adult coral. That said, the genus is known to produce secondary metabolites that act as herbivory deterents and may be considered cytotoxic (Renaud et al., 1990; Ktari & Guyot, 1999). Therefore, allelopathic effects of *Padina spp.* on coral fragments is possible.

*Mortality*

Similar to the pattern observed in growth, heat treatment did not significantly alter coral fragment mortality in any species at any site or depth. There are several explanations for the ineffectiveness of heat treatment. First, the heat stress used in this study may not have been intense enough to illicit an acclimation response among the corals. To observe changes in expression of genes associated with thermal tolerance, Bashis et al (2012) conducted a heat stress experiment where corals were exposed to ~32 ºC over 72 hours. At this temperature, they were able to detect an upregulation of 60 genes associated with increased thermal tolerance. Unfortunately, exposure to this temperature led to bleaching and mortality in one of the species used in their study. Similar studies by DeSalvo et al (2008;2010) quantified differences in gene expression among *A. palmata* and *O. faveolata* respectively when exposed to thermal stress. In these studies, both coral species were exposed to ~32 ºC for 2 and 11 days respectively, with temperatures increased from ambient (~30 ºC) within the first 3 hours of the experiment. *Acropora palmata* fragments bleached on day 1, while *O. faveolata* fragments began bleaching on day 3. With these results in mind, we elected to use a lower acclimation temperature to avoid significant mortality before outplanting. Furthermore, our results from Chapter 1 concluded that acclimation to ~30 ºC was enough to increase the lifespan of *A. cervicornis* fragments introduced to a temperature above their thermal threshold, so we believed adequate thermal stress was

applied to coral fragments to illicit an acclimation response in *A. cervicornis.* Further experimentation will be necessary to determine if 30 ºC is an appropriate temperature to frontload *A. palmata* and *O. faveolata*.

Second, the duration of stress may not have been sufficient to acclimate fragments to elevated thermal conditions. Previous studies have shown that a short term heat stress of 31 ºC for 48 hours increases thermal tolerance 1-2 weeks after the acclimation period (Middlebrook and Gouldbergh, 2008). However, no studies have investigated if short-term heat stress increases thermal tolerance over months or years. Instead, previous research has revealed that the immediate environment surrounding the colony throughout its development plays a large role in accliamation. Acclimation to these "microenvironments" is accomplished through changes in epigenetic patterns (Durante et al., 2019) and gene expression (Seneca et al., 2010). In our experiment, corals were collected from CRF's offshore nursery, which is designed so that coral fragments are suspended from fishing line on PVC trees. This design may alter the amount of sunlight individual fragments receive, with fragments on the base of the tree acclimating to lower irradiance compared to corals attached near the top. Furthermore, CRF colonies were not collected from one reef, but a variety of sites throughout the upper and middle Keys. If long-term acclimation influences the thermal tolerance of individual gentoypes, it is possible corals may exhibit thermal tolerance suitable to their original reef site.

Symbiont composition may be another factor that influenced coral mortality. Certain symbiont clades are more resilient to thermal stress than others (Berkelmans & Van Oppen 2006), so it is possible that different symbiont populations among species or even among genotypes could have helped maintain low mortality among outplants. During monitoring in the August timepoint, it was noted that all *O. faveolata* fragments had changed color from dark

brown to a yellowish-gold, and maintained this coloration throughout the last monitoring date in March 2020. This may be an example of "symbiont shuffling" (Meiog et al, 2007) where background populations of thermally resilient Symbiodiniaceae are retained within the coral's tissues while less tolerant clades are expelled (Cunning et al., 2018; Silverstien et al., 2017). Symbiont shuffling may explain why *O. faveolata* experienced almost no mortality during August and October timepoints, while *A. palmata* exhibited high rates at the same timepoints. It is also possible that this change in coloration may be due to minor bleaching, with the corals never returning to pre-outplant symbiont density. A future analysis of dinoflagellate clade and density is necessary to determine if symbiont shuffling or chronic bleaching explain the change in coloration in *O. faveolata*.

Sedimentation, bleaching, and predation were unlikely contributers to coral mortality in this study. No bleaching was directly observed at any timepoint, although this doesn't preclude bleaching as a contributer to mortality among outplants as bleaching may have occurred in between timepoints. Predation did not significantly contribute to mortality. Two fire worms were found among two *A. cervicornis* fragments and one *A. palmata* fragment in CF October 2018, but no tissue was observed to have been stripped off of the fragments. Only one *O. faveolata* fragment was recorded as "non-viable" in response to parrotfish predation at CF in October 2018.

A singificant proportion of mortality among *A. cervicornis* may be due to White Band Disease (WBD), a common bacterial infection that can be transmitted between corals in the genus *Acropora*. According to Gignoux-Wolfsohn et al., (2012) WBD is transmitted through the water colum and is most prevalent in late summer due to warmer water temperatures. Twelve fragments exhibited signs of the disease in August and 2 fragments exhibited symptoms in

March at CF. Infections occured at random, with G-tests of independence showing no difference in infection among Treated/Shallow, Treated/Deep, Untreated/Shallow, and Untreated/Deep fragments. Although no disease was found among NDR fragments, disease was noticed on other CRF outplants approximately 20 m North of our outplant location, so some NDR corals may have been infected between monitoring timepoints. Despite *A. palmata's* proclivity for acquiring WBD, no signs of the disease were observed on *A. palmata* fragments at any timepoint at either site. While WBD was observed only among *A. cervicornis* at Carysfort, our limiting monitoring may have been insufficient in documenting all cases of the disease over the course of 8 months, so it is possible WBD may have significantly contributed to mortality among *A. cervicornis* and *A. palmata* outplants.

It should be noted that heat treatment may not be beneficial if corals are frontloaded and never encounter elevated heat in the wild. The duration of stressful water temperature (surface temperature ≥ 30 ºC) has varied over the last 10 years in the Florida Keys according to the National Data Buoy Center (NDBC). Data acquired from buoy MLRF1 shows temperatures in 2011, 2014, and 2015 exceeding 30 ºC for 1000 hours or more, while 2018 barely exceeded 200 (National Data Buoy Center, 2018). If treated outplants experienced limited thermal stress on the reef, acclimation to elevated temperatures would have little effect on improving the growth or viability of the coral. Heat acclimation may be detrimental if the environment does not fit the pojected acclimation conditions. Added thermal stress increases production of heat-shock, symbiont-recognition, and structural proteins which allocate resources to pathways associated with acclimation instead of growth and reproduction (Kenkel et al., 2013). This means heat-treated corals may sacrifice growth if they are conditioned to maintain thermal tolerance, particularly if they experience other forms of chronic stress throughout the year (Edmunds &

Davies, 1989). Fortunately, no negative effects were observed as a result of thermal stress among heat treated coral. Therefore, further studies should investigate if thermal frontloading is a viable strategy if enacted prior to a heatwave, as at worst the thermal regime applied in this study produced no difference in the outcome of coral viability or growth.

In conclusion, our study was unable to improve coral outplant growth or viability through thermal "frontloading". There are several parameters that may account of the shortcomings of this experiment. First, our acclimation temperature of 30 $^{o}$C may not be stressful enough to induce frontloading, and higher acclimation temperature may be necessary to initiate long-lasting changes in gene expression. Secondly, frontloading may only be useful if environmental temperatures reach thresholds that stage an advantage to corals acclimated to elevated temperatures, such as the extreme seasonal high recorded in 2015. Furthermore, heat stress may require weeks or months to enact long-term acclimation. If this is true, nursery conditions may play a primary role in determining the acclimation potential of corals to habitats exhibiting elevated temperatures. Laslty, investigating novel techniques that reduce disease and competiton with macroalgae will prove crucial if growth and mortality among outplants are to be improved upon.

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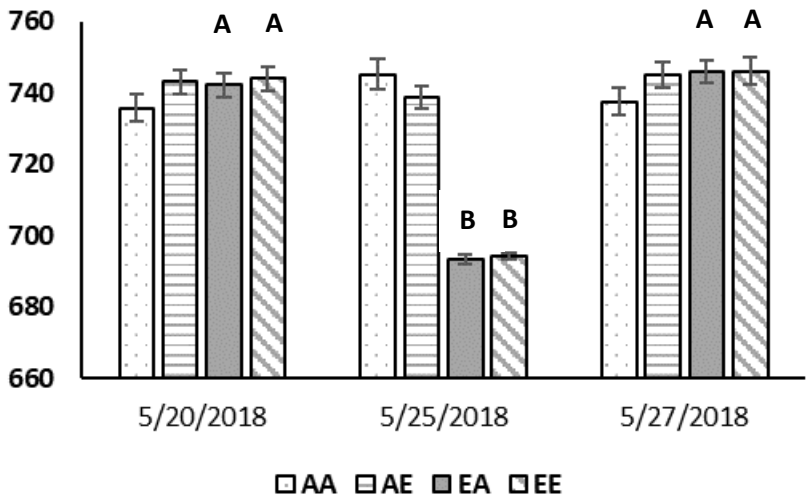


**Figure 1.** Average Fv/Fm (±se) values among four treatments over three timepoints, before heat acclimation (5/20/18), immediately after heat acclimation (5/20/18), and after recovery (5/27/2018). AA includes fragments subjected to ambient acclimation/ambient long-term conditions. Group AE includes fragments subjected to ambient acclimation /elevated long-term conditions. EA includes fragments subjected to elevated acclimation /ambient long-term conditions. EE includes fragments subjected to elevated acclimation /elevated long-term conditions. Letters denote significant differences among groups.

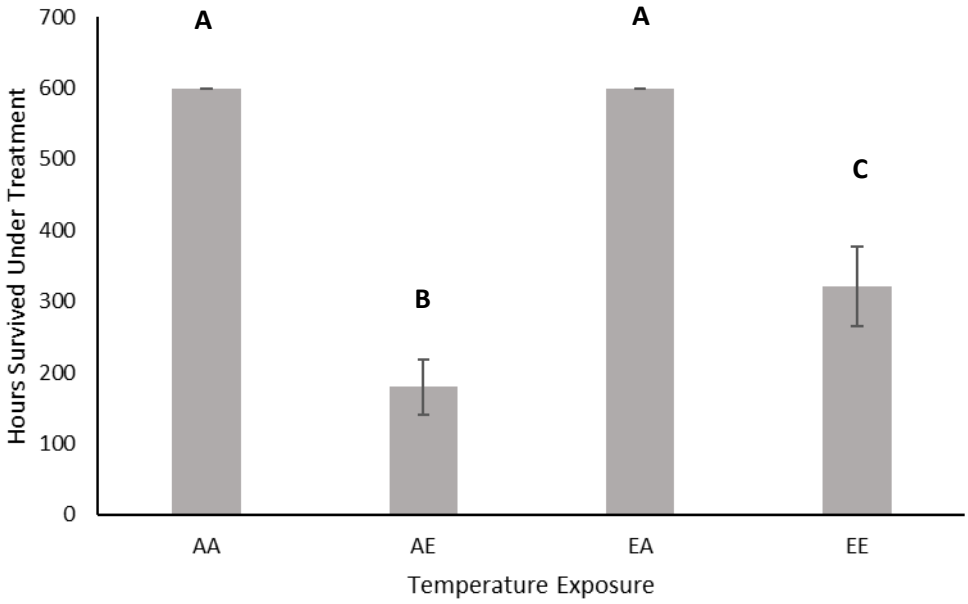


**Figure 2.** Average hours (±se) survived by each coral among four treatments. AA includes fragments subjected to ambient acclimation/ambient long-term conditions. Group AE includes fragments subjected to ambient acclimation /elevated long-term conditions. EA includes fragments subjected to elevated acclimation /ambient long-term conditions. EE includes fragments subjected to elevated acclimation /elevated long-term conditions. All fragments in AA and EA survived for the duration of the experiment. Letters denote significant differences in hours survived.

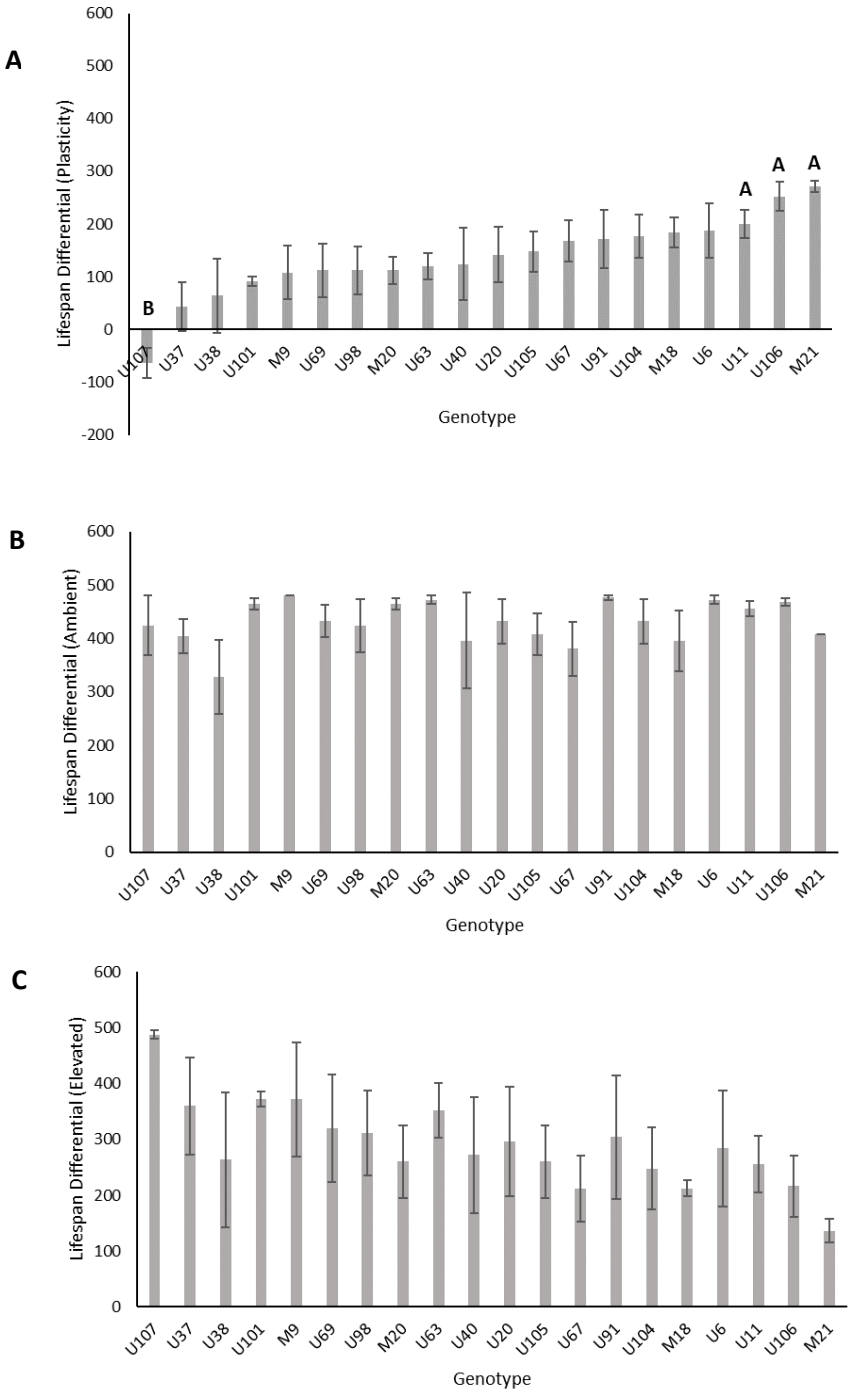


**Figure 3. A)** Average difference (±se) in hours survived under treatment between ambient and heat acclimated fragments for each genotype. **B)** Average difference (±se) in hours survived of heat treated and control fragments that had been acclimated to ambient temperature. **C)** Average difference (±se) in hours survived of heat treated and control fragments that had been acclimated to elevated temperature. In all cases, different letters above bars denote significant differences in lifespan differentials among genotypes.

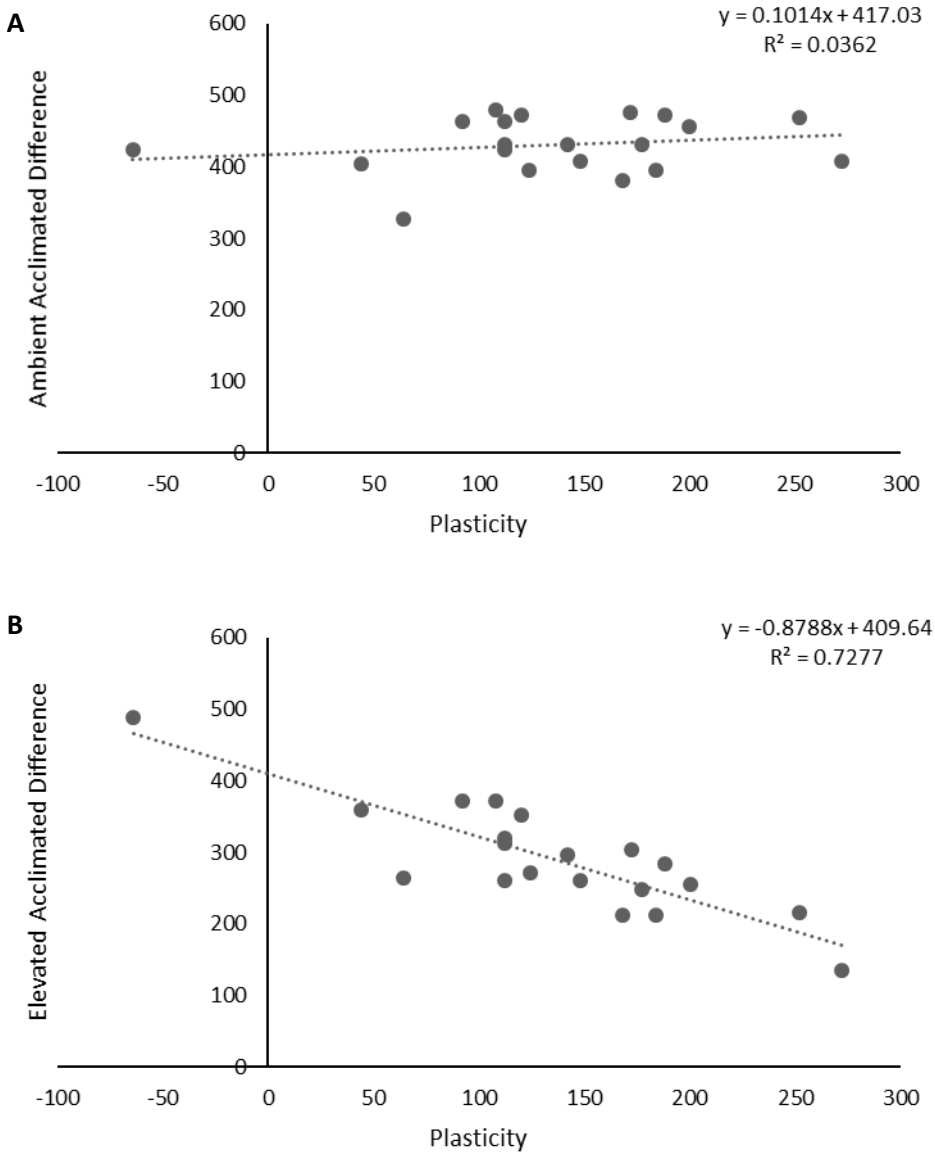


**Figure 4.** A) Regression of the average thermal tolerance of genotypes when acclimated at ambient temperatures (27 °C) vs. the average amount of plasticity in thermal tolerance of a genotype. B) Regression of average thermal tolerance of genotypes when acclimated at elevated temperature (30 °C) vs. the average amount of plasticity in thermal tolerance of a genotype. Thermal tolerance in each case was defined as the difference in lifespan between coral fragments under thermal stress and controls. Plasticity of thermal tolerance was defined as the difference between thermal tolerance when acclimated at ambient temperature and thermal tolerance when acclimated to elevated temperature.

**Table 1.** Seven different estimators of pairwise relatedness among fragments were utilized to estimate the narrow sense heritability ($h^2$) for three different categories of thermal tolerance: 1) plasticity (the difference between 2 and 3), 2) when corals were acclimated to ambient temperature, and 3) when corals were acclimated to elevated temperature.

| | Variance in Relatedness | Plasticity | | Ambient | | Elevated | |
|---|---|---|---|---|---|---|---|
| Estimator | | $h^2$ | Std. Error | $h^2$ | Std. Error | $h^2$ | Std. Error |
| TrioML | 0.01885 | 0.005 | 0.1377 | 0.0061 | 0.1376 | 0.0002 | 0.138 |
| Wang | 0.09385 | 0.012 | 0.3061 | 0.0004 | 0.3079 | 0.0015 | 0.3078 |
| LynchLi | 0.11267 | 0.013 | 0.3352 | 0.0002 | 0.3374 | 0.0005 | 0.3374 |
| LynchRd | 0.02465 | 0.004 | 0.1575 | 3.89E-05 | 0.1578 | 5.00E-05 | 0.1578 |
| Ritland | 0.06986 | 0.003 | 0.2654 | 0.0002 | 0.2657 | 0.0006 | 0.2656 |
| QuellerGT | 0.05107 | 0.011 | 0.226 | 0.0002 | 0.2272 | 0.0009 | 0.2271 |
| DyadML | 0.02068 | 0.003 | 0.1443 | 0.0042 | 0.1443 | 0.0003 | 0.1445 |

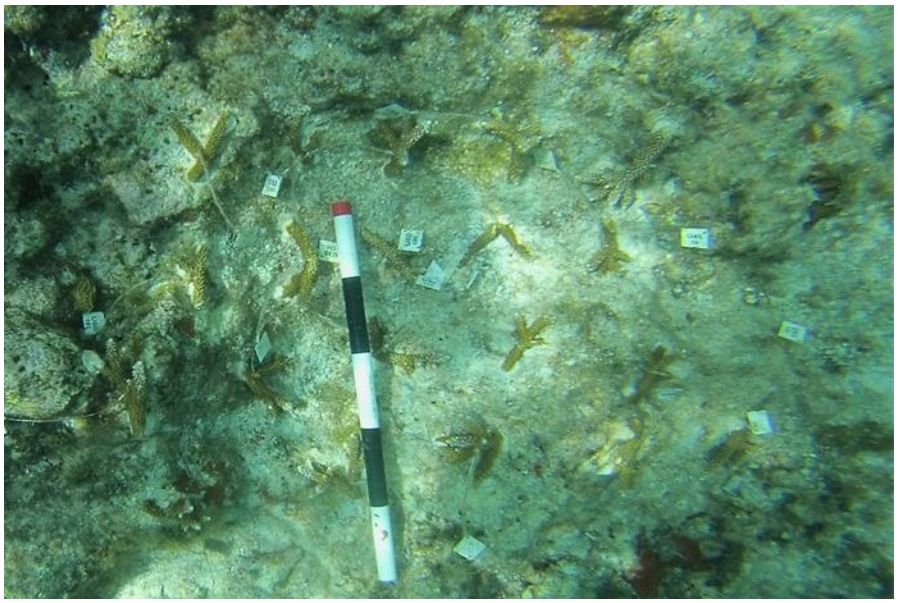

**Figure 5**. A photograph of a quadrat of *Acropora cervicornis* arranged in a randomized grid design attached to the ocean floor using a two-part marine epoxy at Carysfort Reef. All corals were outplanted in six quadrats, with 3 attached in a ~3 m ledge, and the other 3 attached to a ~6 m ledge.

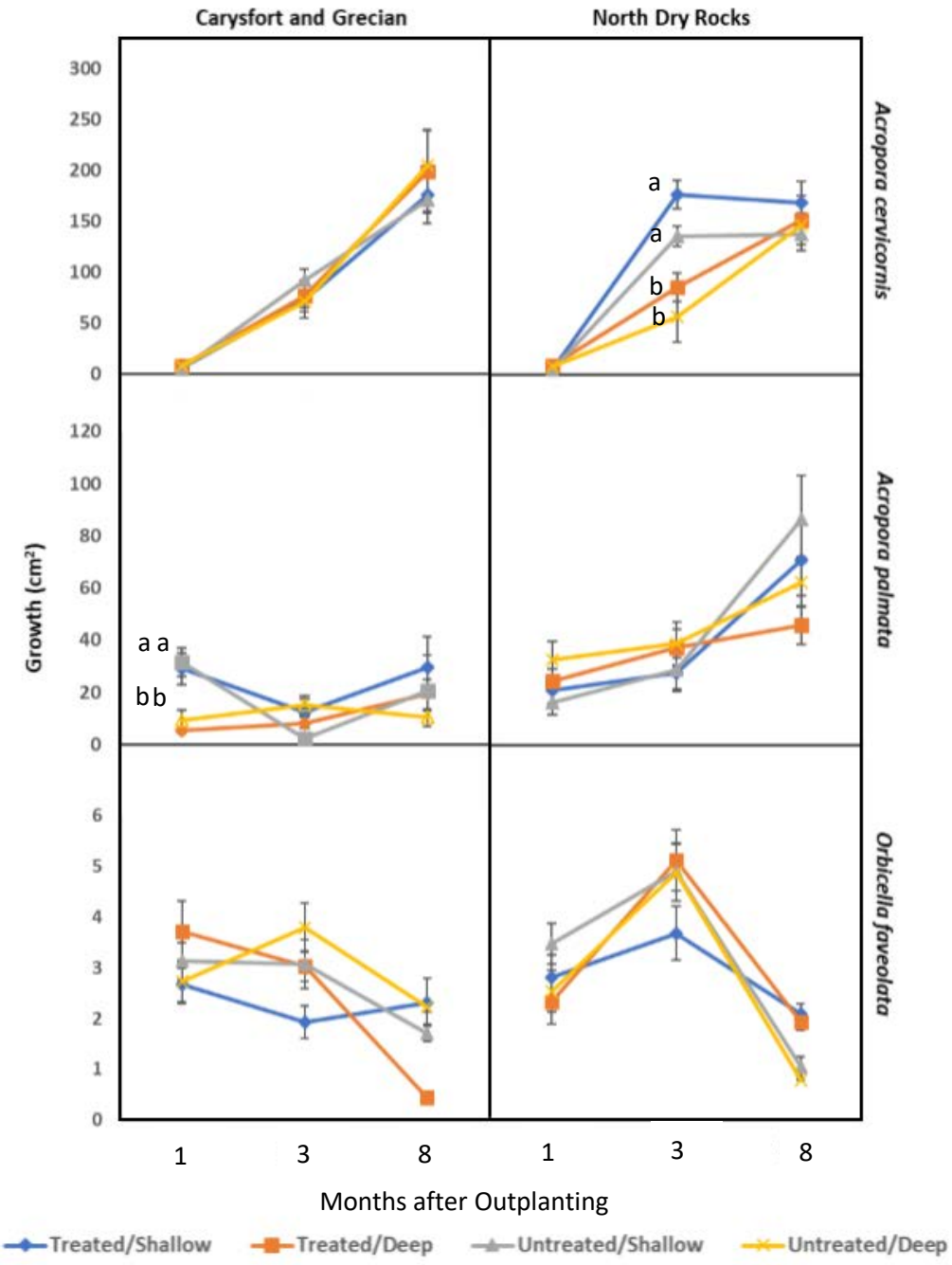


**Figure 6.** The average difference in surface area of outplanted coral fragments among three timepoints: 1, 3 and 8 months after outplanting. Growth is shown in 4 groups: Treated/Shallow, Treated/Deep, Untreated/Shallow, and Untreated/Deep. Treated corals received a heat stress of 30° C over 48 hours before outplanting. Corals were outplanted at 2 depths, ~3 m labelled as "shallow" and ~6 m as "deep". Lower case letters depict significantly different groups within one timepoint.

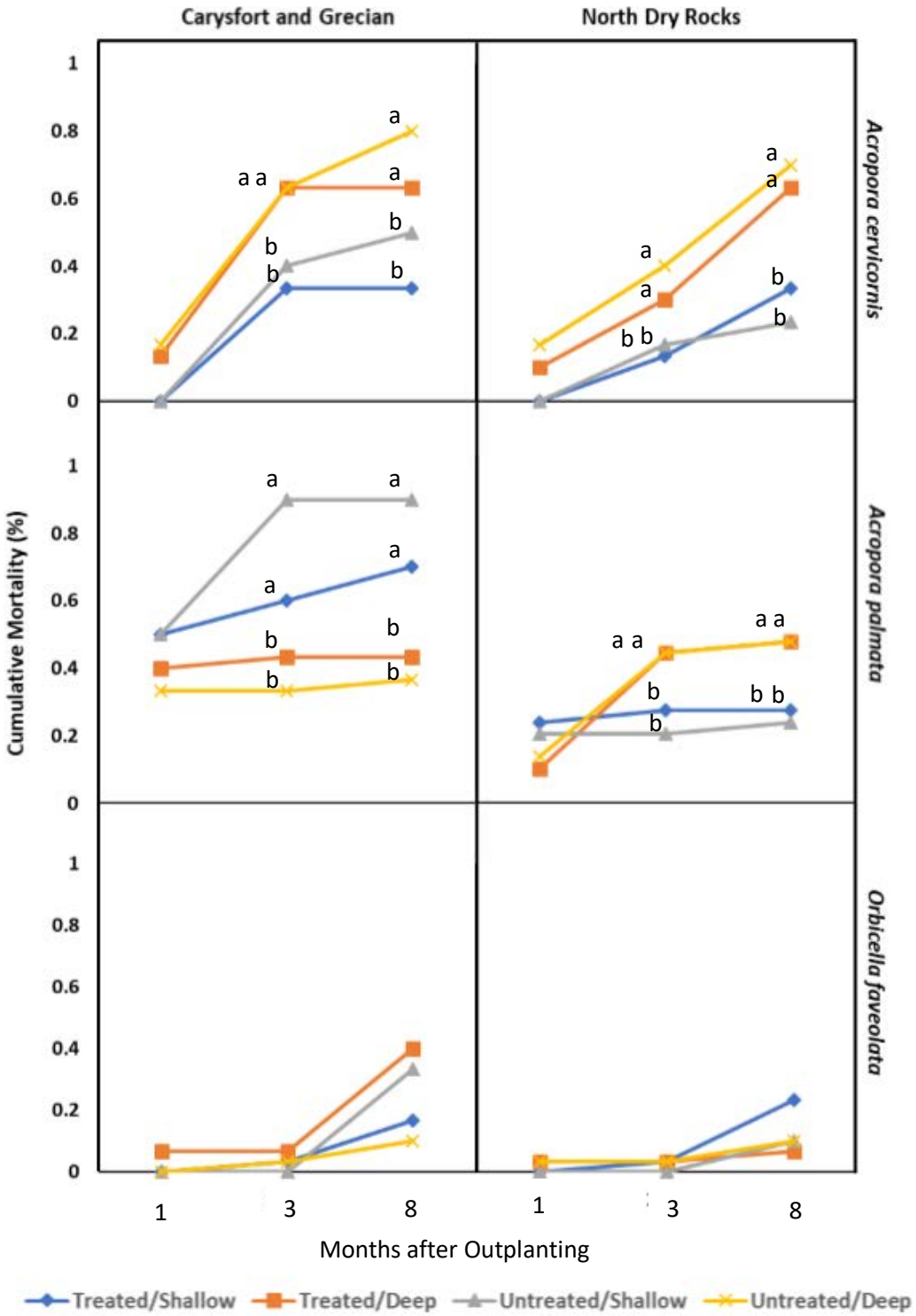


**Figure 7.** Cumulative percent mortality of *A. cervicornis*, *A. palmata*, and *O. faveolata* over 8 months after outplanting. Total mortality counts of each species were taken at three timepoints after outplanting: 1 month, 3 months, and 5 months. Coral fragments were divided into four groups: Treated/Shallow, Treated/Deep, Untreated/Shallow, and Untreated/Deep. Treated corals received a heat stress of 30° C over 48 hours before outplanting. Corals were outplanted at 2 depths, ~3 m labelled as "shallow" and ~6 m as "deep". Lowercase letters denote significant differences in mortality within each timepoint.